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The role of arsenate reducing bacteria for their prospective application in arsenic contaminated groundwater aquifer system

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ABSTRACT

Arsenate reducing bacteria are known to potentially contribute to metabolize or transform arsenic from groundwater or sediments. Environmental arsenic bioavailability and the influence of these bacteria on arsenic mobilization have not been comprehensively investigated till date. In this present study, *Bacillus sp.* BAR1, isolated from the severely arsenic contaminated groundwater of Bhojpur, Bihar within the middle Indo – Gangetic plain were thoroughly characterized for its detailed physiological, biochemical and phylogenetic analysis. It was revealed to be a gram-positive heterotrophic bacteria, capable of utilizing arsenate as an electron acceptor under aerobic conditions. The strain showed a broad range of tolerance to several heavy metals and transition metal ions (Hg, Zn, Cr, Cu, Cd, Ni) including arsenic and utilized several carbon sources along with complex sugars. The growth of the isolate also substantially increased in the presence of arsenate, signifying the utilization of arsenate as an added energetic advantage during the detoxification mechanism of arsenate to arsenite. The arsenate reduction efficiency was optimized to 72% at a temperature of 30 °C, inoculum 2%, an initial As (V) concentration of 100 mM at 20 h using response surface methodology. The results shed light on the key role of *Bacillus sp.* BAR1 in the self-purification of heavy metal-laden waters and in the arsenic biogeochemical cycle.

1. Introduction

Toxic arsenic (As) in alluvial groundwater of South East Asia; particularly Bihar, West Bengal, Assam, and Bangladesh has polluted the major drinking water resources, distressing billions of people. Arsenic-rich aquifer sediments are primarily responsible for groundwater As contamination in the region (Ohtsuka et al., 2013). Subsurface geomicrobiological activity within the geochemical cycle is also responsible for prevalent groundwater As contamination (Zhu et al., 2014). Microbial arsenate (As V) reduction takes place via two mechanisms; dissimilatory reduction and detoxification. Cytoplasmic arsenate reductase *ArsC* catalyzes the detoxification of As (V) into arsenite (As III) besides extruding it outside the cell using an efflux pump (Silver and Phung, 2005). In sub-oxic alluvial groundwater, the most feasible As mobilization mechanism is the bacterial transformation of sediment-bound As (V) within host minerals such as arsenopyrite (Osborne et al., 2015). During anaerobic metabolism, As (V) as a terminal electron acceptor is used by some dissimilatory As (V) reducing bacteria, reducing it into a more soluble (As (III)) species (Kudo et al., 2013). Although this has been a subject of substantial interest, the prominent function of As (V) reducing bacteria in the discharge of As in alluvial environments remain sufficiently unexplored.

Low nutrient content has been characterized within the middle Gangetic plains of West Bengal and Bihar. The As rich aquifer in the region has a high content of alkane hydrocarbons and the bacteria utilize As (V) as the sole carbon source (Ghosh et al., 2015).Till date, 21 cultivable isolates have been studied with respect to physiological and taxonomic characterization of As (V) reducing bacteria (Osborne et al., 2015). ***Bacillus***, ***Wolinella***, ***Citrobacter***, ***Clostridium***, ***Sulfurospirillum***, and *Desulfomicrobium*etc have been identified and classified as major As (V) reducers (Silver and Phung, 2005). Reduction of As (V) into mobile and more toxic As (III) leads to environmental As dissemination via microbial As (V) respiration. Bacterial reduction of As (V) is mostly carried out in the aqueous phase (Oremland and Stolz, 2005). Arsenic removal from contaminated groundwater is supported by these transformations under specific environmental conditions. Co-precipitation of crystalline, stable As (III) sulfide occurs by some dissimilatory As (V) reducing strains in the presence of thiosulphate and As (V) ions (Drewniak et al., 2015). Hence such micro-organisms are primarily responsible for the purification of groundwater. The middle Gangetic plains remain adequately unexplored, as only a few As (V) reducing strains have been studied till date, limiting the knowledge of these bacteria in the As

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mobilization and biogeochemical cycle. In this study, a detailed characterization of As (V) reducing *Bacillus sp.* BAR1 has been done to determine insight into the tolerance and adaptation capabilities of the isolate to regulate its authentic role in As biogeochemical cycle.

2. Materials and methods

2.1. Enrichment, isolation and enumeration of as-resistant bacteria

The collection of groundwater sample, having total As concentration of 4.76 mg/l was done from Cheenegaon village (25.6869780N, 84.6366080E) in Bhojpur, Bihar. The bacterial population numbers were enriched by inoculating 100 ml of the sample in MSM liquid media (Himedia, India) enriched with 25 mM of sodium arsenate (Sarkar et al., 2013). Succeeding As (V) amendment, the samples were incubated at 30 °C and 180 rpm for 14 days in the dark. Serial dilution up to 10^{-8} was done for the As (V) enriched cultures ensuring bacterial growth, following plating on MSM agar media. The plates were then incubated for 10 days at 30 °C. Arsenic resistant ten bacterial forming units were selected at definite time intervals after three enrichment cycles, and stored for further analysis as purified glycerol stocks. Furthermore, the As (V) reduction ability of the isolates was tested using 5 mM of sodium arsenate. A yellow colored precipitate of Ag_3AsO_3 (Silver orthoarsenite) on flooding the plates with 0.1 M silver nitrate ($AgNO_3$) confirmed the reducing nature of the strains (Simeonova et al., 2004).

2.2. Heavy metal tolerance of the isolates

Agar dilution method was used to test the maximum tolerable concentration (MTC) of the selected isolates in addition to other heavy-metals including As. Graded concentrations of As (V) ($Na_2HAsO_4.7H_2O$) and As (III) ($NaAsO_2$) were amended up to 100–800 mM and 15–70 mM in MSM agar plates. Furthermore, the strains were tested for other heavy metals and transition metal ions including KCr_2O_7, $HgCl_2$, $ZnCl_2$, $NiCl_2.6H_2O$, $CuCl_2$ and $CdCl_2$ (Merck, Germany) in minimal salt (MSM) agar plates with variable concentrations of 2.5–30 mM. An inoculum size of 1% was used to aseptically inoculate the strains on the metal-amended plates, followed by incubation at 30 °C for 48 h.

2.3. Physiological and biochemical characterization of the test isolates

Gram staining was performed using the standard gram staining kit (Himedia, India). A bright field microscope was used to observe the test isolate's cellular structure. An Environmental Scanning Electron Microscopy (ESEM, Quanta FEC 250, Pfeiffer, Netherland) was used to characterize the surface structure, colony size, and appearance. The pH sensitivity and temperature was monitored at varying pH (5–9) and incubation temperatures (4 °C - 45 °C). Standard antibiotic discs (Himedia, India) and carbohydrate discs (25 μg sugar/disk) were used to assess the varied antibiotic sensitivity and metabolizing capability of the isolates on phenol red agar plates. Biochemical properties were tested viz. Voges-Proskauer's test, methyl red test, citrate utilization test, and indole test in accordance to manufactures protocol (Himedia, India).

2.4. Reductive transformation of the test isolates

A qualitative approach such as silver nitrate screening assay was used to validate the reducing nature of the isolates (Bagade et al., 2016). As (V) reduction of the test isolate was observed along with the growth of the isolate in MSM media. The optical density was measured spectroscopically and the triplicate samples were collected every 1 h. For quantitative estimation, the reduced As (V) concentration was measured using the spectrophotometric As (V) molybdenum blue complex (Dhar et al., 2004). The strain showing the highest efficiency was further considered for its molecular identification and apparent factor optimization.

2.5. Molecular identification and phylogenetic analysis

A FastDNA Spin kit was used to extract the genomic DNA from the As (V) reducing test strain using the manufacture's protocol (Himedia, India). The template for PCR amplification was the extracted genomic DNA. The amplification of the 16S rRNA gene was done using universal forward primer, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer1492R (5′ TACGGTTACCTTGTTACGACTT-3′). The initial denaturation and melting was done for 5 min and 30 s at 95 °C, annealing at 55 °C for 30 s and followed by extension at 72 °C for 1 min 30 s (Biswas et al., 2019). The finishing extension step was done at 72 °C for 10 min. Following 30 cycles, separation of the amplified product was done in 0.7% agarose Tris-acetate EDTA (TAE) gel by electrophoresis and a UV trans-illuminator was used to visualize the amplified bands (Biswas and Sarkar, 2018). Eurofins sequencing service (Eurofins Genomics India Pvt. Ltd., Bangalore, India) performed the sequencing of 16S rRNA gene. Furthermore, the processing of the sequences was done by means of BioEdit tool (BioEdit v7.0.5). The homology sequences were then obtained from the National Center for Biotechnology Information (NCBI) BLAST program. CLUSTALX software was used to align the related reference sequences and the 16S rRNA sequence retrieved from the universal GenBank database. Finally, the construction of the phylogenetic tree was done using the neighbor-joining method in MEGA X (Kumar et al., 2008).

2.6. Optimization of process parameters for As (V) reduction using response surface methodology

The As (V) reduction by the selected test strain was optimized using response surface methodology (RSM). A 2-level central composite design (CDD) was implemented in this study bearing four factors such as time (10 h–30 h), initial As (V) concentration (100 mM–500 mM), temperature (30 °C–37 °C), and inoculum percentage (1% - 3%) based on experimental conclusions. The final concentration of As (V) was availed as the sole response constituent. An overall 30 set of runs or experiments were generated. The designed set of experiments was carried out using DESIGN EXPERT 10.0 software (Free trial).

Analysis of Variance (ANOVA) determines the effect of the numerous factors on the response, following which the model equations related them with independent variables. The analysis of variance also assesses the error sum of squares (SSE), regression sum of square (SSR), and the total corrected sum of squares (SST). The fitting of the model is regulated by the R^2 value or the coefficient of determination. The hypothesis testing is done by the F-test and P-value signifies the result reliability. In this current study, 95% confidence level was presumed to be significant. Finally, a quadratic regression equation was obtained based on the results and the estimation of the R^2 value was done for this individual model.

3. Results and discussion

3.1. Isolation of As (V) reducing bacterial isolates

A total of ten morphologically distinct As-resistant bacterial strains were sequestered from the groundwater sample of Bhojpur, Bihar. The silver nitrate screening assay further confirmed the reducing nature of the strains. Decoupling occurs through As (V) and Fe (III) reduction, causing the discharge of As into the groundwater. The metabolically active microbial communities undergo dynamic changes causing adaptation of different respiratory pathways via altered expression of metal reductases (Gault et al., 2003). Intensive use of phosphate fertilizers also causes mobilization of sorbed As, causing its discharge into the groundwater system (Harvey et al., 2002). Two strains namely,

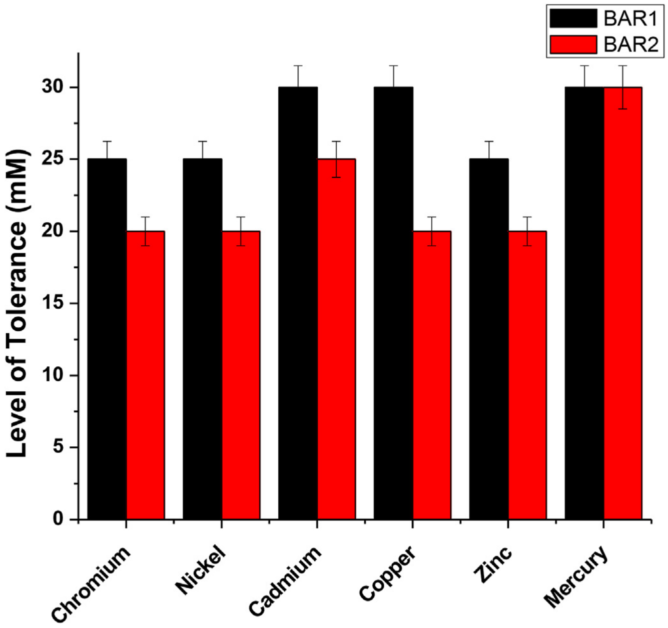

Fig. 1. Heavy metal resistance of the isolates.

BAR1 and BAR2 were chosen for further characterization based on their superior As resistance capabilities.

3.2. *Arsenic and other heavy metal resistance of the test isolates*

The isolates showed high multi-metal resistance, including As (III), As (V) and other heavy and transition metals up to 70 mM, 500 mM, and 30 mM concentration (Fig. 1). The genetic and the physiological makeup aids the metal resistance ability of the microbes, useful for As remediation from polluted sites. The exopolysaccharides on the bacterial cell surface are known to form a complex with the adsorbed heavy metals, allowing them to survive under stress conditions, serving as a chief mechanism of detoxification (Anwanyu and Ugwul, 2010). Several bacterial phyla including *Firmicutes*, *proteobacteria,* and *actinobacteria* have been reported for their high multi-metal resistance (Sheik et al., 2012). A variety of detoxification mechanisms also attributes to the heavy metal resistance by the microbes including exopolysaccharide complexation, metal efflux and metal reduction (Bowman et al., 2018). Enhanced tolerance to heavy metals, causes these bacteria to advance energy and upsurge their rate of growth during major respiratory transformations (Biswas et al., 2017). Prolonged exposure to different species of highly concentrated metals in groundwater possibly aspects to stress-induced metal resistance, making them potent prospective agents for As contaminated groundwater bioremediation.

3.3. *Physiological and biochemical characterization of the selected isolates*

The physiological characterization showed the isolates to be of mesophilic (30 °C) nature, surviving under mildly acidic conditions (pH - 6.8). Previously, several As resistant bacteria have been reported to have the same mesophilic nature of survival (Guo and Chen, 2017). Strains BAR1 and BAR2 were seen to be rod-shaped and gram-positive in colony morphology. Both the isolates were negative to indole test and positive to Methyl-Red and Voges-Proskauer's test, concluding that the strains could perform mixed acid fermentation and produce acetoin. Strain BAR1 could not utilize citrate as the sole carbon source while BAR2 showed positive for the same. The strains also utilized oxidase to an extensive range. The resistance properties towards antibiotics within the test strains seemed like a stimulating outcome. The isolates exhibited medium to superior sensitivity to Vancomycin, Doxycycline hydrochloride, Rifampicin, Metronidazole, Ampicillin, Norfloxacin, Neomycin, Gentamicin, Tetracycline, Ciprofloxacin, Ceftazidime, and Trimethoprim. while poor sensitivity towards Cefadroxil, Roxithromycin, and Cefotaxime. The isolates utilized carbohydrates efficiently including all complex multi-carbons including Sucrose, Raffinose, Lactose, Fructose, Mannitol, Sorbitol, Salicin, Maltose, Melibiose, Inositol, Sucrose, Mannose, Galactose, and Xylose except Arabinose. The use of alternate organic carbons aids the bacteria to survive within the challenging inherent habitats (Sarkar et al., 2014).

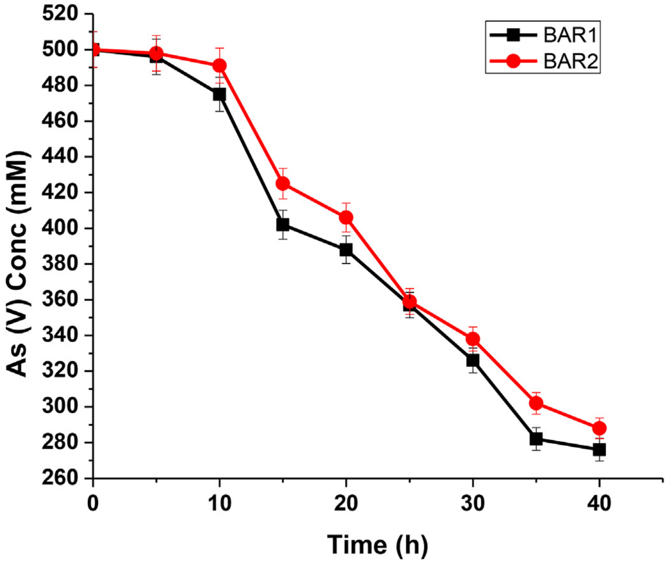

Fig. 2. Reduction kinetics of the isolates.

3.4. *As (V) reduction assay*

The As (V) reducing nature of both the strains was confirmed using silver nitrate assay. Strain BAR1 and BAR2 showed a significant reductive As (V) transformation of 44.8% and 42.4% from an initial As (V) concentration of 500 mM within 40 h of growth (Fig. 2). Among the two strains, BAR1 showed comparatively higher reduction ability and was further studied for its growth kinetics. The growth rate for BAR1 in the presence of As (V) was 1.5 h^{-1} with a doubling time of 0.46 h and in the absence of As (V) was 1.4 h^{-1} with a doubling time of 0.50 h (Fig. 3). With an initial increase in the As (V) concentration, strain

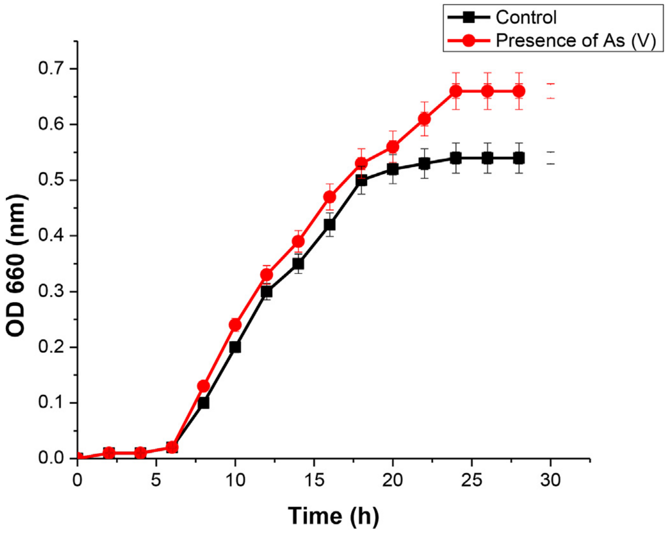

Fig. 3. Growth kinetics of the test isolate BAR1.

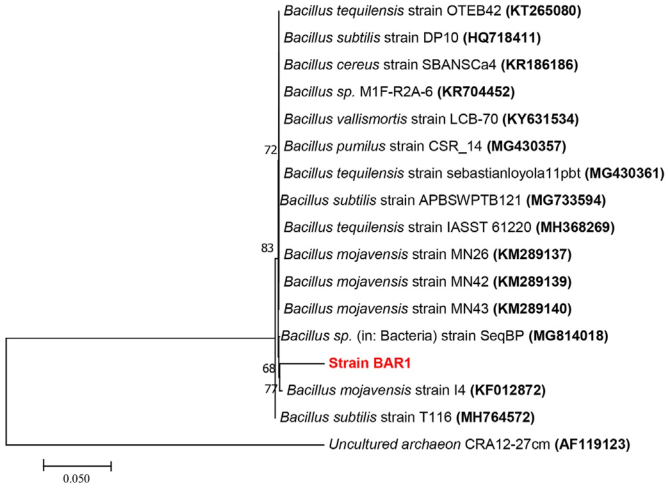

Fig. 4. Phylogenetic tree of Bacillus sp. BAR1 based on 16S rRNA sequences.

BAR1 seemed to gain an energetic advantage, owing to the presence of an external As (V) concentration. Similar to prokaryotic molybdenum inclosing polypeptides (e.g. formate dehydrogenases N and H in *Escherichia coli*), arsenate reductase catalyzes the reduction mechanism of As (V) to As (III) during bacterial growth. Independent *arsB*, *arsC* or other novel pathways could mediate the As (V) transformation within the bacterium (Chen et al., 2017). Reports suggest that the presence of excess hydrogen ions and the arrangement of *ars* operon in numerous bacterial genera may also have a stimulatory effect on As transformation under specific conditions (Zhang et al., 2015).

3.5. Phylogenetic analysis of the test strain

The phylogenetic analysis of the amplified 16S rRNA gene of the superior As (V) reducing strain BAR1 revealed its homology with numerous *Bacillus sp*; specifically, *Bacillus mojavensis,* belonging to the *Bacillaceae* family (Fig. 4). Analysis usingneighbor-joining based method, bared its similarity with numerous other *Bacillus sp*. such as *Bacillus mojavensis* strain I4 (KF012872), *Bacillus tequilensis* strain IASST 61220 (MH368269), *Bacillus subtilis* strain APBSWPTB121 (MG733594), *Bacillus pumilus* strain CSR_14 (MG430357) and *Bacillus vallismortis* strain LCB-70 (KY631534) with a bootstrap value of greater than 65%. *Bacillus mojavensis* has been previously reported to be highly halotolerant besides having flocculent properties (Guo and Chen, 2017). Hence, its multi-metal tolerance capacity can aid in the mobilization, transformation, and removal of toxic As from groundwater as incremental microbial stimulators (Cao et al., 2015).

3.6. Optimization of As (III) oxidation during growth

The optimization of the reduction of As (V) was performed using a 2 level central composite design by response surface methodology. An

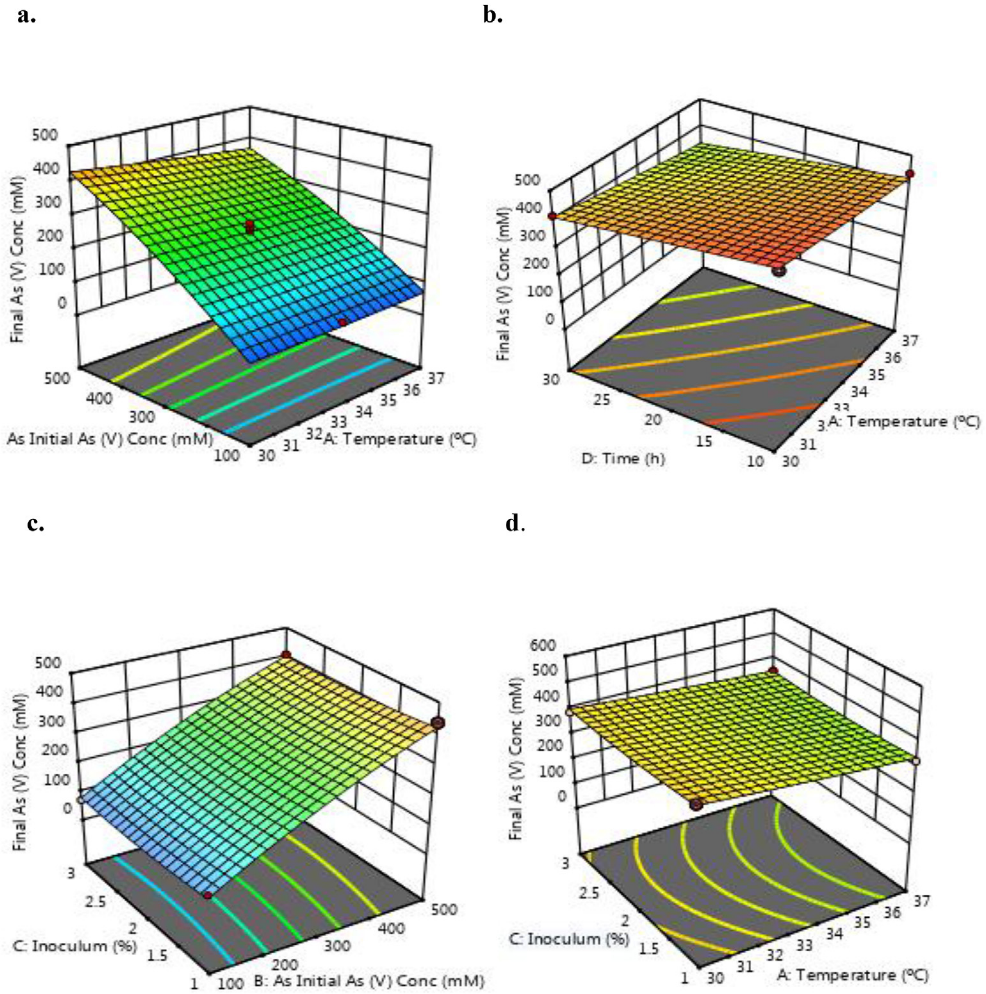

Fig. 5. 3-D contour plots for As (V) reduction w.r.t (a) Initial As (V) Concentration and temperature (b) Time and temperature (c) Inoculum and Initial As (V) Concentration (d) Inoculum and temperature.

Table 1
Analysis of Variance of the experimental design for As (V) reduction.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5.573E+05	14	39804.64	105.18	< 0.0001	significant
A-Temperature	4264.83	1	4264.83	11.27	0.0043	
B–As Initial As (V) Conc	4.958E+05	1	4.958E+05	1310.04	< 0.0001	
C-Inoculum	411.79	1	411.79	1.09	0.3134	
D-Time	6610.15	1	6610.15	17.47	0.0008	
AB	3921.52	1	3921.52	10.36	0.0057	
AC	405.69	1	405.69	1.07	0.3169	
AD	36.63	1	36.63	0.0968	0.7600	
BC	129.98	1	129.98	0.3435	0.5666	
BD	4077.76	1	4077.76	10.78	0.0050	
CD	777.20	1	777.20	2.05	0.1723	
A^2	66.82	1	66.82	0.1766	0.6803	
B^2	7066.76	1	7066.76	18.67	0.0006	
C^2	3222.05	1	3222.05	8.51	0.0106	
D^2	1.13	1	1.13	0.0030	0.9572	
Residual	5676.52	15	378.43			
Lack of Fit	3737.19	10	373.72	0.9635	0.5533	not significant
Pure Error	1939.33	5	387.87			
Cor Total	5.629E+05	29				

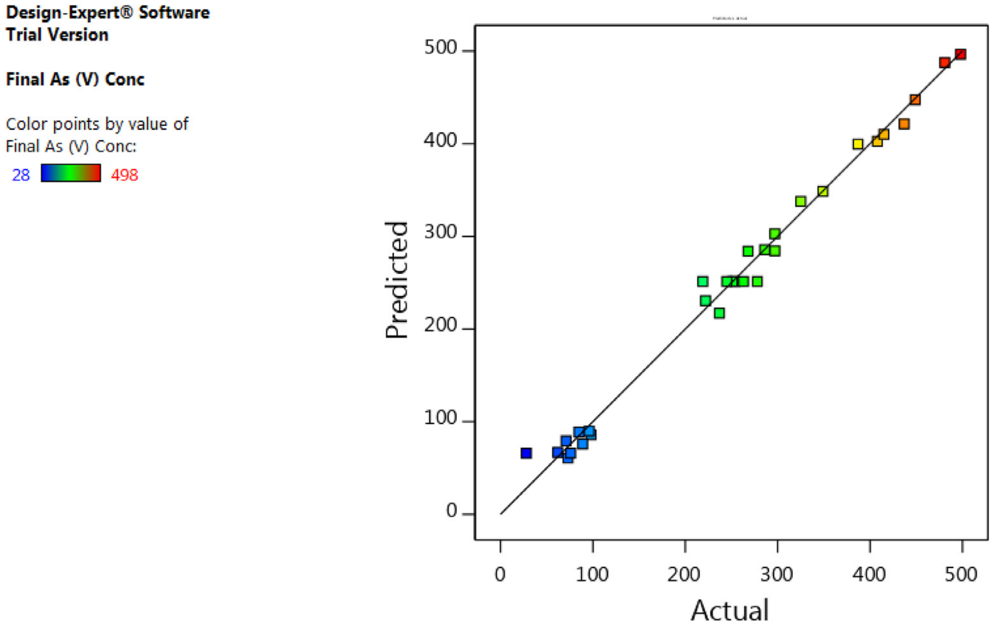

Fig. 6. Actual Vs predicted graph for As (V) reduction.

illustrative contour plot for bio-reduction of As (V) combining the effect of As (V) concentration and temperature, time with temperature, inoculum with initial As (V) Concentration and temperature is demonstrated in Fig. 5 (a), (b), (c) and (d). The initial As (V) concentration had a slight negative effect on the reduction potential of the strain i.e. under similar operating conditions; the reduction transformation to As (III) was sufficiently low. Reactive oxidative species may be generated as the As (III) ions attach itself to the bacterial cell wall that causes cell destruction by oxidizing the lipid and the cellular proteins (Yan et al., 2014). Contrastingly, inoculum percentage and time had a positive influence on the reduction potential of As (V) up to a certain limit i.e 2% and 20 h. Similarly, bio-transformation of As (V) slackened with increasing temperature. The best optimum bio-reduction efficiency was observed with an inoculum 2%, at a temperature of 30 °C, initial As (V) concentration of 100 mM at 20 h following inoculation. A neutral to alkaline nature of the operating conditions enhance the growth of bacteria. Moderate alkalinity and strongly reducing conditions facilitate the release of As (Huq et al., 2018).

The ANOVA data clearly indicated that the quadratic model had an extensive effect on the generated responses (Table 1). The R^2 value for the final As (V) concentration was 0.9899 designating that the model can predict significantly 98.99% of the complete generated experimental data. Factors having a p-value less than 0.05 have an operative impact on the response. The ANOVA data reveals that A, B, D, AB, BD, B^2, and C^2 have a positive effect on the reduction potential of As (V). The quadratic equations which relate the variables with the response are represented in Eqn (1).

$$\textbf{As (V) Reduction} = 251.33 – 13.44\ A + 164.40\ B – 4.32\ C – 16.73\ D - 15.84\ AB + 5.35\ AC – 1.53\ AD – 3.03\ BC – 16.16\ BD + 7.40\ CD + 1.55\ A^2 – 20.90\ B^2 + 10.73\ C^2 – 0.2010\ D^2 \quad (1)$$

Based on the model equation and the experimental data, the predicted versus actual data for the final As (V) concentration is shown in Fig. 6. It was seen that the experimental data agreed well with the model predicted values in most of the area. For the final As (V) concentration, the model could not explain about 0.02% data due to slender over-extrapolation in the higher section of the responses. This may be due to the non-contemplation of third order interactions and cubic terms in the generated model equation.

3.6.1. Optimization and validation of the model

Optimized conditions can be derived from the obtained regression equation considering a targeted As (III) reduction of 50% within a set range of factors using response surface methodology. Different goals were set for the response and the independent variables in a numerical optimization process to set the optimum conditions. Based on these values, different conditions were provided. Among the set optimum conditions, one optimized condition was used to validate the model using an As (V) concentration of 100 mM, temperature of 30 °C, inoculum 2%, and time 30 h. It was observed that almost 72% As (V) reduction was perceived at the end of 30 h, postulating that the regression model succeeds in elucidating the complete process with judicious precision.

4. Conclusion

Extensive studies have been done over the past decade onto the processes regulating the release of As into the groundwater systems, but it still remains a matter of strong debate. Microbial reduction of sorbed As (V) into more mobile As (III), and dissolution of oxy-hydroxide phases are some of the proposed mobilization mechanisms (Das et al., 2004). In this study, a highly resistant bacterial isolate *Bacillus sp*. BAR1 was isolated from the severely As contaminated aquifer of Bhojpur, Bihar, capable of environmentally significant As bio-transformations. Such indigenous microbial communities can effectively aid in designing of an in-situ bioremediation technology within the contaminated sites. Moreover, the As (V) reducing activity by *Bacillus sp*. has not been reported in the literature to date. Hence, this study paves the way for a more novel and prospective application for the biological treatment of As-contaminated aquifers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101218.

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